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Cinnamate-4-Hydroxylase Expression in Arabidopsis[1]

Regulation in Response to Development and the Environment

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Cinnamate-4-hydroxylase (C4H) is the first Cyt P450-dependent monooxygenase of the phenylpropanoid pathway. To study the expression of this gene in *Arabidopsis thaliana*, a C4H cDNA clone from the Arabidopsis expressed sequence tag database was identified and used to isolate its corresponding genomic clone. The entire C4H coding sequence plus 2.9 kb of its promoter were isolated on a 5.4-kb *Hin*dIII fragment of this cosmid. Inspection of the promoter sequence revealed the presence of a number of putative regulatory motifs previously identified in the promoters of other phenylpropanoid pathway genes. The expression of C4H was analyzed by RNA blot hybridization analysis and in transgenic Arabidopsis carrying a C4H-β-glucuronidase transcriptional fusion. C4H message accumulation was light-dependent, but was detectable even in dark-grown seedlings. Consistent with these data, C4H mRNA was accumulated to light-grown levels in etiolated *det1–1* mutant seedlings. C4H is widely expressed in various Arabidopsis tissues, particularly in roots and cells undergoing lignification. The C4H-driven β-glucuronidase expression accurately reflected the tissue-specificity and wound-inducibility of the C4H promoter indicated by RNA blot hybridization analysis. A modest increase in C4H expression was observed in the *tt8* mutant of Arabidopsis.

The phenylpropanoid pathway gives rise to a wide array of metabolites. These compounds participate in many plant-defense responses (Nicholson and Hammerschmidt, 1992) and absorb potentially damaging UV-B radiation (Caldwell et al., 1983; Li et al., 1993; Landry et al., 1995). The pathway also generates the monomers required for lignin biosynthesis: ferulic acid and sinapic acid (Lewis and Yamamoto, 1990). The purification of the soluble enzymes of the phenylpropanoid pathway over the last 10 years has permitted the cloning of their respective genes. These include the genes encoding Phe ammonia-lyase, *p*-coumarate CoA ligase, caffeic acid/5-hydroxyferulic acid, *O*-methyltransferase, chalcone synthase, and chalcone isomerase. In comparison, the genes encoding two of the Cyt P450-dependent monooxygenases (P450s) in this pathway, C4H and ferulate-5-hydroxylase, were more difficult targets for identification because these proteins are relatively unstable, are of low abundance, and are membrane-bound. Despite these difficulties, the gene encoding C4H was identified (Fahrendorf and Dixon, 1993; Mizutani et al., 1993; Teutsch et al., 1993) following purification of the enzyme from Jerusalem artichoke and mung bean (Gabriac et al., 1991; Mizutani et al., 1993). The gene encoding ferulate-5-hydroxylase was recently cloned by T-DNA tagging (Meyer et al., 1996b), an approach that circumvented the requirement for protein purification.

Given the importance of the many pathways that include P450-catalyzed steps (Bolwell et al., 1994; Durst and O'Keefe, 1995; Schuler, 1996), it is likely that these proteins and the factors that regulate the expression of their corresponding genes have a key role in the biochemistry of plants. For example, C4H introduces the phenolic hydroxyl that is necessary for the laccase- or peroxidase-catalyzed activation of monolignols to their corresponding quinone methides during lignification (Lewis and Yamamoto, 1990; O'Malley et al., 1993). The hydroxylation catalyzed by C4H also shifts the UV absorbance spectrum of cinnamate toward the longer wavelengths that are absorbed by *p*-coumarate and its subsequent metabolites, thus providing plants with compounds that are effective at absorbing biologically relevant wavelengths of UV light (Landry et al., 1995).

The cloning of cDNAs encoding phenylpropanoid pathway P450s provides the opportunity to integrate studies on their regulation with previous studies on phenylpropanoid pathway gene expression. For example, C4H activity is induced by a number of stimuli, including light, elicitors, and wounding (Russell, 1971; Beneviste et al., 1977; Bolwell and Dixon, 1986). More recent studies have documented increases in C4H mRNA abundance in response to some of the same factors (Fahrendorf and Dixon, 1993; Buell and Somerville, 1995; Logemann et al., 1995). In addition, C4H activity is modulated in response to the application of exogenous *p*-coumaric acid (Lamb and Rubery, 1976), suggesting that the endogenous pools of phenylpropanoid intermediates may play an important role in the regulation of C4H gene expression, as has been documented for other genes in this pathway (Bolwell et al., 1988; Loake et al., 1992; Orr et al., 1993).

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Abbreviations: C4H, cinnamate-4-hydroxylase; EST, expressed sequence tag.

To study the expression of C4H in Arabidopsis we have identified a cDNA clone from the Arabidopsis EST database (Newman et al., 1994; Cooke et al., 1996) that encodes the Arabidopsis ortholog of C4H. We have sequenced this cDNA and used it to analyze C4H expression by RNA blot hybridization analysis and to isolate a cosmid clone that carries the C4H genomic sequence. We have sequenced this genomic clone to characterize the structure of the C4H locus in Arabidopsis, to identify putative regulatory sequences found upstream of the coding sequence, and to generate a C4H-reporter gene construct to evaluate the tissue-specificity and inducibility of the promoter. We have also examined Arabidopsis mutants defective at various steps in the phenylpropanoid pathway to determine whether C4H expression is altered in these mutants.

MATERIALS AND METHODS

Arabidopsis thaliana L. Heynh. ecotypes Columbia or Landsberg *erecta* were cultivated at a light intensity of 100 μE m^{-2} s^{-1} at 23°C under a photoperiod of 16 h light/8 h dark in ProMix (Premier Horticulture Inc., Red Hill, PA) potting mixture. For seedling plant material to be used in the analysis of C4H mRNA accumulation, seeds were surface-sterilized for 30 min in a 2:1 mixture of 0.1% Triton X-100 and household bleach. Seeds were rinsed thoroughly with sterile water and plated on Miracloth (Calbiochem) discs on modified Murashige-Skoog medium (ammonia-free medium to which an additional 20.6 mM potassium nitrate was added in place of ammonium nitrate) (Murashige and Skoog, 1962) containing 0.7% agar.

DNA and RNA Blotting

For Southern blot analysis, DNA was extracted from leaf material (Rogers and Bendich, 1985) digested with restriction endonucleases, electrophoretically separated, transferred to Hybond N^+ membrane (Amersham), and hybridized with cDNA probes according to standard protocols (Sambrook et al., 1989). Blots were washed twice for 15 min at 65°C in 2× SSPE, 0.1% SDS, followed by a single 15-min wash at 65°C in 0.2× SSPE, 0.1% SDS, and were then exposed to film. RNA was extracted from leaf material (Goldsbrough and Cullis, 1981), electrophoretically separated, transferred to Hybond N^+ membrane, and probed with radiolabeled probes prepared from cDNA clones according to standard protocols.

Identification of cDNA and Genomic Clones

The C4H cDNA EST clone 126E1T7 was obtained from the Ohio State Arabidopsis Resource Center (Columbus). C4H genomic clones were isolated from a Landsberg *erecta* genomic library generated in the binary cosmid vector pBIC20 (Meyer et al., 1996a) using a radiolabeled probe derived from the cDNA insert of EST clone 126E1T7. A 5.4-kb *Hin*dIII DNA fragment containing the entire C4H coding sequence from one of the cosmids was subcloned into pGEM-7Zf(+) (Promega) in both the 5′-3′ and 3′-5′ orientation and transformed into *Escherichia coli* DH5α. Nested deletions for both cDNA and genomic clones were made in both 5′ and 3′ orientations using the Promega Erase-a-Base kit according to manufacturer's recommendations. Useful nested deletion clones were identified by size selection using PCR. Sequence analysis was performed on plasmid DNA by manual sequencing using the United States Biochemical Sequenase kit version 2.0 or by automated sequencing on an Applied Biosystems 373A DNA sequencer.

Determination of C4H Map Position

The position of the C4H locus within the Arabidopsis genome was determined by restriction fragment length polymorphism mapping among the recombinant inbred lines generated from the Columbia and Landsberg *erecta* ecotypes (Lister and Dean, 1993). DNA isolated from 79 recombinant inbred lines was scored by Southern blot analysis using a *Sty*I polymorphism identified between the two parental lines.

Determination of Transcription Start Site

The transcription start site of the C4H gene was determined by primer extension using an oligonucleotide (5′-CCATTATAGTTTGTGTATCCGC-3′) complementary to the 5′ end of the C4H cDNA clone. This oligonucleotide was end-labeled with [γ-^{32}P]ATP using polynucleotide kinase, and an amount of labeled primer equaling 400,000 cpm was added to 20 μg of total RNA isolated from Arabidopsis stems, precipitated, and dried. The DNA-RNA hybrids were dissolved in 30 μL of hybridization buffer (80% formamide, 1 mM EDTA, 0.4 M NaCl, 14 mM Pipes, pH 6.4), incubated at 85°C for 10 min and at 28°C overnight, and reprecipitated. The dried pellet was resuspended in 20 μL of reverse transcriptase buffer, and the primer was extended using Moloney murine leukemia virus reverse transcriptase (Gibco). The extended product was analyzed by gel electrophoresis, adjacent to the products of a sequencing reaction performed with the primer extension oligonucleotide and the C4H genomic clone.

Construction of the C4H-GUS Fusion

A C4H-GUS transcriptional fusion was constructed using a 2897-bp C4H promoter nested deletion clone carrying the C4H transcription start site. The 3′ end of the selected clone terminated at position +34 within the region corresponding to the 5′ untranslated region of the C4H cDNA. This fragment was liberated from pGEM-7Zf(+) by digestion with *Hin*dIII and *Apa*I and was subcloned into *Hin*dIII-*Sma*I-digested pBI101 using an *Apa*I-blunt-ended adapter. Ligation products were transformed into *E. coli* NM544. The recombinant plasmids were characterized by diagnostic restriction digests prior to use in plant transformation experiments.

Plant Transformation

Constructs for plant transformation were introduced into *Agrobacterium tumefaciens* C58 pGV3850 (Zambrisky et al., 1983) by electroporation. Plant transformation was per-

formed by vacuum infiltration (Bent et al., 1994) with minor modifications. Briefly, a 500-mL culture of transformed *Agrobacterium* harboring the C4H-GUS fusion construct was grown to stationary phase in Luria broth containing 10 mg L^{-1} rifampicin and 50 mg L^{-1} kanamycin. Cells were harvested by centrifugation and resuspended in 1 L of infiltration media containing 2.2 g of Murashige-Skoog salts (Murashige and Skoog, 1962), Gamborg's B5 vitamins (Gamborg et al., 1968), 0.5 g of Mes, 50 g of Suc, 44 nM benzylaminopurine, and 200 μL of Silwet L-77 (OSI Specialties, Charleston, WV) at pH 5.7. Bolting Arabidopsis plants (T_0 generation) that were 5 to 10 cm tall were inverted into the bacterial suspension and exposed to a vacuum (>500 mm of Hg) for 3 to 5 min. Infiltrated plants were returned to standard growth conditions for seed production. Transformed seedlings (T_1) were identified by selection on Murashige-Skoog medium containing 50 mg L^{-1} kanamycin and 200 mg L^{-1} Timentin (SmithKline Beecham, Philadelphia, PA) and were transferred to soil.

GUS Assays

Tissues from kanamycin-resistant T_1 Arabidopsis plants were incubated in a solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc), 100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% (v/v) Triton X-100 from 8 to 12 h at 37°C (Stomp, 1992). Tissues were destained three times in 70% ethanol and whole mounts and sections were analyzed by bright-field microscopy.

RESULTS

Identification of the Arabidopsis C4H cDNA Clone

A search of the Arabidopsis EST library using the keyword "cinnamate" identified a number of clones, most of which corresponded to members of the Cyt P450 gene superfamily. One of these sequences (clone ID no. 126E1T7, GenBank accession no. T44874) was clearly homologous to the C4H sequences that were characterized from mung bean and Jerusalem artichoke (Mizutani et al., 1993; Teutsch et al., 1993). This clone also appeared to be a full-length P450 cDNA. The putative C4H cDNA was sequenced and was found to be 69 to 72% identical to the C4H sequences available in the database, and its deduced amino acid sequence shares 84 to 86% identity. The Arabidopsis C4H cDNA encodes a protein of 57,798 D. Its inferred amino acid sequence suggests that, like many other P450s, it possesses a short N-terminal signal peptide followed by a hydrophobic region that probably constitutes a single-pass transmembrane helix. This domain is followed by the sequence PPGPIPVP, which obeys the consensus sequence P/IPGPXG/PXP, which is thought to play an essential role in the orientation of the P450 relative to the membrane with which it is associated (Szczesna-Skorupa et al., 1993; Yamazaki et al., 1993). The sequence PFGVGRRSCPG, found near the carboxy terminus of the Arabidopsis C4H, is identical to that of other C4H proteins and obeys the consensus PFGXGRRXCXG for the conserved heme-binding domain of Group A plant P450s (Durst and O'Keefe, 1995). Several regions of amino acid sequence divergence are found when the Arabidopsis and other C4H sequences are compared. In particular, conservative substitutions are found in the N-terminal hydrophobic transmembrane domain and significant sequence divergence can be seen in domains that may correspond to the D, H, and J helices of other P450s (Von Wachenfeldt and Johnson, 1995).

Recently, an independently isolated Arabidopsis cDNA, in which the deduced amino acid sequence is identical to the sequence reported in this paper, has been cloned and heterologously expressed in yeast (P. Urban and D. Pompon, personal communication), confirming the identity of the C4H cDNA reported here.

Identification of the Arabidopsis C4H Genomic Clone

To evaluate whether C4H is encoded at a single locus in Arabidopsis, the C4H cDNA was used as a probe against Arabidopsis DNA digested with a number of restriction enzymes (Fig. 1). The probe hybridized to a single band in all lanes except those containing the *Xma*I and *Sty*I digests, consistent with the presence of sites for these enzymes within the cDNA. Comparison of the hybridization banding pattern obtained with Columbia and Landsberg *erecta* DNA identified a restriction fragment length polymorphism with *Sty*I. This polymorphism permitted the mapping of the C4H gene to the lower arm of chromosome 2 using recombinant inbred populations (Lister and Dean, 1993). The C4H locus maps to a position 0.8 cM below the marker m283c and 5.1 cM above the marker m323. Further evidence that C4H is encoded by a single

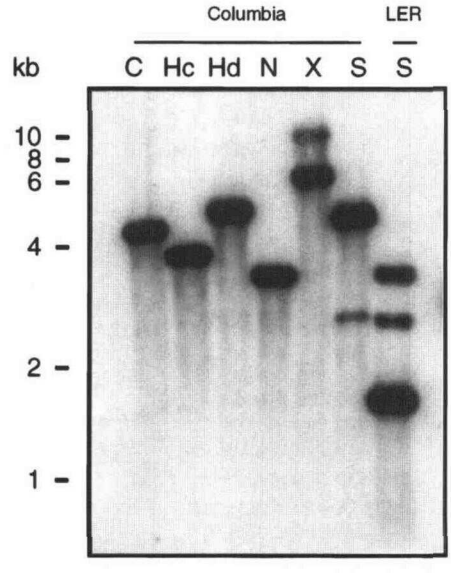

Figure 1. Genomic Southern blot analysis of the C4H locus in Arabidopsis. The C4H cDNA was used as a probe against DNA isolated from the Columbia ecotype digested with *Csp*45I (C), *Hin*cII (Hc), *Hin*dIII (Hd), *Nde*I (N), and *Xma*I (X). DNA from both Columbia and Landsberg *erecta* ecotypes digested with *Sty*I (S) was included to illustrate the restriction fragment length polymorphism identified with this enzyme.

gene in Arabidopsis was provided by searching the *A. thaliana* EST database with the full-length C4H cDNA sequence. This search retrieved the EST reported here as well as for four other sequences (GenBank accession nos. F19837, T04086, N65601, and T43776) that are essentially identical to the full-length C4H cDNA sequence. The similarity of the C4H cDNA sequence to all others in the database is substantially less after these five are considered. This suggests that there are no other closely related C4H-like genes expressed in Arabidopsis.

Using the C4H cDNA as a probe, a genomic cosmid library was screened to identify a C4H genomic clone. Twelve overlapping genomic clones that covered the C4H locus were isolated, and restriction analysis revealed that these clones fell into three different classes. Southern blot analysis indicated that each clone contained a *Hin*dIII fragment that carried the entire C4H coding sequence. This 5.4-kb *Hin*dIII fragment was subcloned from one of the cosmids and sequenced. Alignment of the genomic sequence with the cDNA revealed that the subcloned fragment carried approximately 3 kb of upstream regulatory sequence, and that the C4H coding sequence is interrupted by two small introns (intron I, 85 bp; intron II, 220 bp) (Fig. 2). The nucleotide sequences at the 5′ exon-intron boundaries are GAA GTGAGT for intron 1 and CCG G-TAACT for intron 2. The respective 3′ splice sites are CAG GC and CAG CG. These sequences agree reasonably well with the consensus Arabidopsis 5′ and 3′ intron/exon boundaries, (C/A)AG GTAAGT and CAG GT, respectively, compiled by Brown et al. (1996).

The C4H cDNA and genomic clones were retrieved from libraries constructed from the Columbia and Landsberg *erecta* ecotypes, respectively. This difference provided the opportunity to compare the sequences of these C4H alleles. Comparison of the two coding sequences revealed 13 nucleotide differences between Columbia and Landsberg *erecta*, 2 of which occur in the first position of codons, and 10 in the wobble positions. All of these changes are silent with regard to the encoded protein. The remaining change (CAC to CTC) results in the substitution of a Leu residue in the Columbia C4H in place of His-92, which is found in Landsberg *erecta* and all other C4H sequences available in GenBank. One of the silent changes results in the conversion of the sequence at positions 3212 to 3217 from CAAAGG in the Columbia allele to CCAAGG in the Landsberg *erecta* allele. Only the Landsberg *erecta* sequence conforms to the restriction site consensus of *Sty*I, which accounts for the restriction fragment length polymorphism generated with this enzyme.

The transcription start site for the C4H mRNA was determined to be 86 bp upstream of the initiator ATG. A putative TATA box is found 33 bp upstream of the transcription start site, and a putative CAAT box at −152. Inspection of the C4H promoter revealed a number of elements that may influence C4H expression. Three elements were identified with the consensus sequence ACCTA (Fig. 2), which is found in the AC-I, AC-III, box 1, box 2, and box L elements of various phenylpropanoid pathway promoters (Ohl et al., 1990; Hauffe et al., 1991; da Costa e Silva et al., 1993; Feuillet et al., 1995; Hatton et al., 1995; Logemann et al., 1995; Wanner et al., 1995). The ACCTA-containing sequence most proximal to the C4H transcription initiation site overlaps the sequence CACGTA, which is similar to the G-box sequence CACGTG, which was previously identified in other light-inducible promoters (Giuliano et al., 1988; Feinbaum et al., 1991; Loake et al., 1992). A similar sequence motif, CACGTT, is found at −148 in the C4H promoter, where it immediately follows the putative CAAT box. At −243 the C4H promoter also contains the sequence CCTACC(N)$_{11}$CT, which is similar to the H-box sequence CCTACC(N)$_7$CT (Lois et al., 1989; Loake et al., 1991, 1992). A box-3-like element (Ohl et al., 1990) with the sequence AACCAACCAA is present at −55, 22 bp upstream of the putative TATA box.

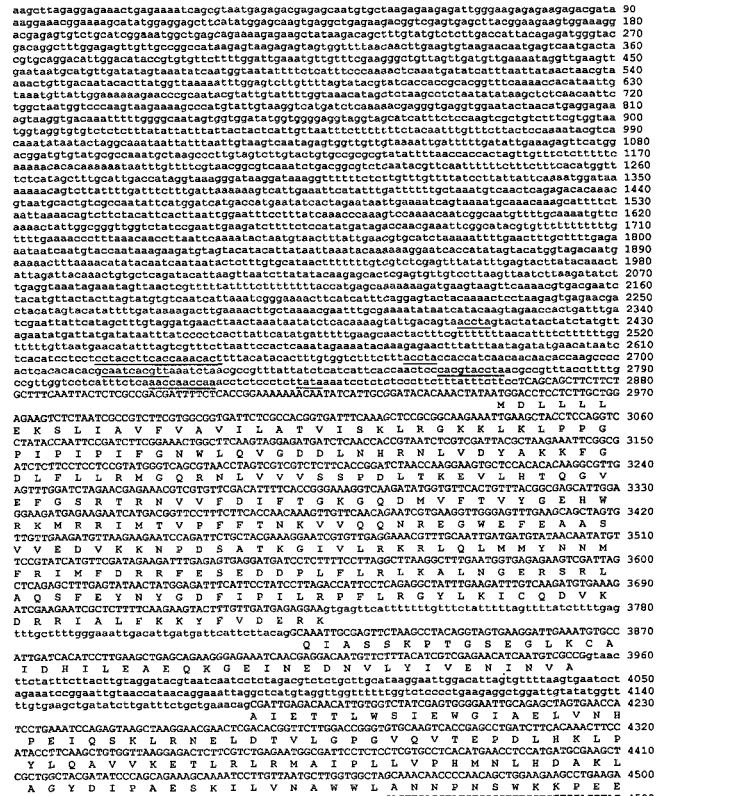

Figure 2. Nucleotide sequence of the Arabidopsis C4H genomic clone and inferred amino acid sequence of the Arabidopsis C4H protein. The nucleotides corresponding to the C4H cDNA clone are shown in uppercase letters with the inferred amino acid sequence indicated below as their single-letter abbreviations. Introns as well as 5′ and 3′ untranscribed/untranslated sequences are shown in lowercase letters. Putative regulatory sequences, including the presumed TATA and CAAT boxes, are underlined.

Analysis of C4H Expression

RNA blot hybridization revealed that C4H mRNA was expressed in all of the tissues that were examined (Fig. 3A). The highest levels were found in stems and roots. To examine whether *C4H* expression in young seedlings was light-dependent, wild-type seedlings were grown for 8 d in constant darkness or under a 16 h light/8 h dark photoperiod, and steady-state levels of C4H mRNA were evaluated (Fig. 3B). RNA extracted from Arabidopsis seeds contained readily detectable levels of C4H message and this level was substantially enhanced in light-grown seedlings. RNA extracted from etiolated seedlings contained lower levels of C4H mRNA than seeds and light-grown seedlings, suggesting that C4H mRNA accumulation is light-dependent. Transfer of etiolated seedlings to light conditions led to a rapid increase of C4H message to levels comparable to those of light-grown seedlings. To further examine the impact of light on C4H message accumulation, blots were prepared from RNA extracted from light- and dark-grown *det1–1* seedlings. In each case, C4H mRNA levels were comparable to those of light-grown, wild-type seedlings.

To evaluate the impact of wounding on C4H expression, mature leaves were sliced repeatedly with a razor blade and were incubated for 48 h on wet filter paper in Petri plates to prevent dessication prior to RNA extraction. Control leaves were incubated similarly but were not wounded. RNA blots prepared from these leaves indicated a strong induction of C4H mRNA in the wounded treatments (Fig. 3C).

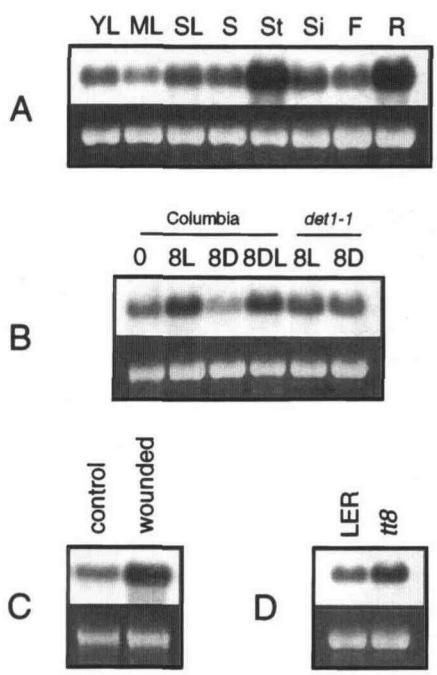

Figure 3. A, RNA blot hybridization analysis of C4H expression in Arabidopsis. The C4H cDNA was used as a probe against RNA isolated from young leaves (YL), mature leaves (ML), senescent leaves (SL), 10-d-old seedlings (S), stems (St), siliques (Si), flowers (F), and roots (R). B, Recently imbibed seeds (0), 8-d-old light-grown seedlings (8L), 8-d-old etiolated seedlings (8D), 8-d-old seedlings that had been grown under dark conditions until d 6 when they were transferred to the light (8DL). C, Mature leaves (control) and mature leaves 48 h after wounding (wounded). D, Eight-day-old seedlings from the wild-type Landsberg *erecta* ecotype and the *tt8* mutant. In each case, 5 μg of RNA was loaded into each lane, and ethidium bromide staining of ribosomal RNAs (lower panels) was used to confirm equivalent loading.

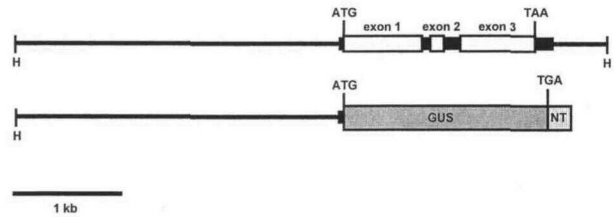

Figure 4. The structure of the C4H gene in Arabidopsis and the GUS construct that was used to evaluate the tissue specificity of C4H expression.

A number of mutants in Arabidopsis are known to be defective in genes encoding enzymes of the phenylpropanoid pathway, or in *trans*-acting factors required for their expression. To test the hypothesis that phenylpropanoid pathway mutations may directly or indirectly affect the transcription of the C4H gene, blot hybridization analyses were conducted using RNA extracted from 8-d-old seedlings from the Columbia and Landsberg *erecta* ecotypes and the mutants *tt1*, *tt2*, *tt3*, *tt4*, *tt5*, *tt6*, *tt7*, *tt8*, *tt9*, *tt10*, *ttg*, *fah1–2*, and *sng1–1*. These experiments indicated that the perturbations in phenylpropanoid metabolism in these seedlings resulted in only modest changes in C4H expression (data not shown). The most substantial change in C4H mRNA abundance was seen in the *tt8* mutant seedlings (Fig. 3D), where C4H message accumulation was nearly doubled (1.87 ± 0.16-fold, $n = 3$) as determined by direct counting of the blots on a InstantImager (model A20240, Packard, Downers Grove, IL).

Tissue-Specific Expression of C4H-GUS Fusions in Transgenic Arabidopsis

To evaluate the tissue specificity of C4H gene expression, a C4H-GUS transcriptional fusion was constructed (Fig. 4) and was used to transform wild-type Arabidopsis. Among a large number of T1 transformant seedlings carrying the C4H-GUS transcriptional fusion, GUS staining patterns were observed (Fig. 5) that were consistent with the RNA blot data obtained using the C4H cDNA probe (Fig. 3A). In cotyledons, GUS staining was diffusely distributed throughout the epidermis and mesophyll with higher levels of staining localized to the vascular tissue and the surrounding parenchyma (Fig. 5A). Strong staining was also seen in structures at the cotyledonary margins that resemble hydathodes. In the meristematic region of the seedling, strong GUS activity was present in the developing primary leaves, where staining was diffusely distributed and was not localized to the developing vascular tissue. The highest level of GUS staining in the seedling was observed in the root. This high level of GUS staining was relatively clearly demarcated beginning at the hypo-

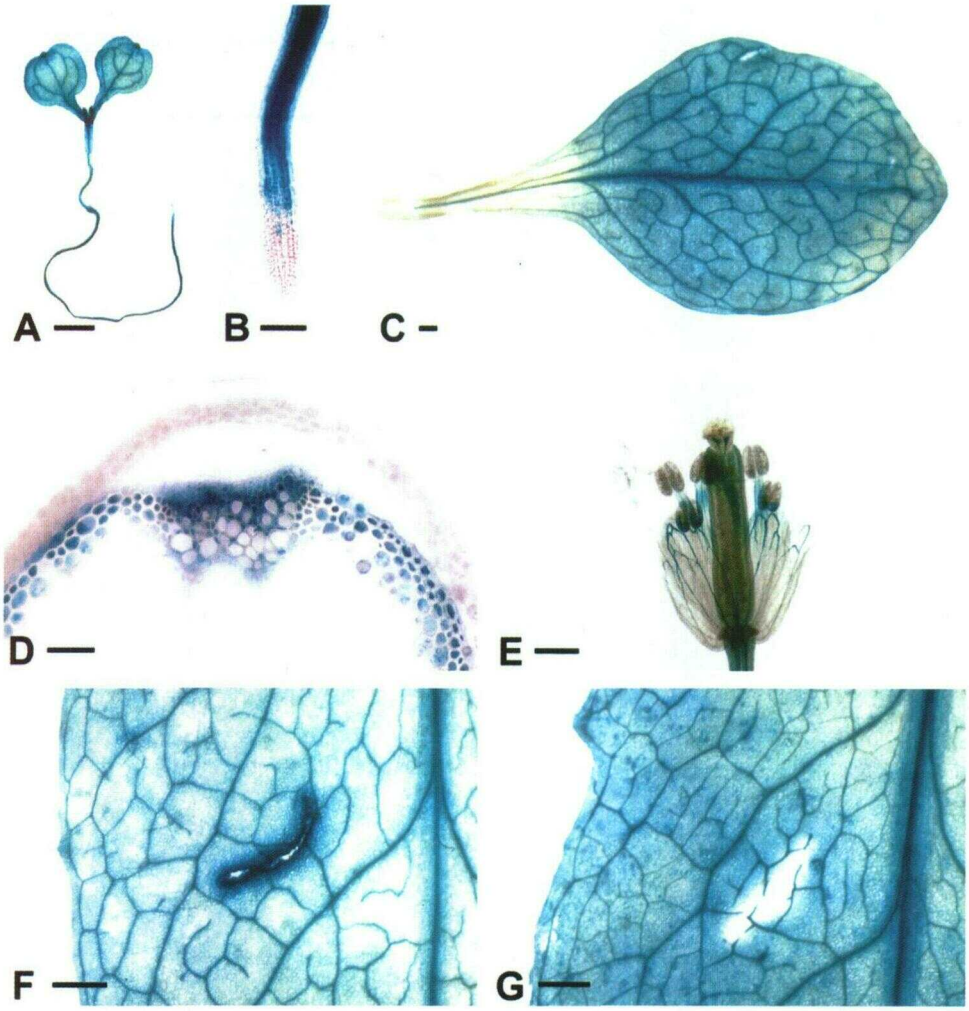

Figure 5. In vivo GUS staining in C4H-GUS transformants. A, Ten-day-old seedling; B, 10-d-old seedling root; C, mature leaf; D, rachis transverse section; E, flower; F, mature leaf stained 48 h after wounding; and G, mature leaf stained immediately after wounding. A, C, E, F, G, Bar = 500 μm. B, D, Bar = 10 μm.

cotyl/root junction, and continuing to near the root tip (Fig. 5, A and B).

In mature leaves GUS staining was strongly localized to the veins (Fig. 5C). Similarly, expression of GUS activity in stem cross-sections was restricted to the xylem and the sclerified parenchyma that extends between the vascular bundles (Fig. 5D). In reproductive tissues weak GUS staining was seen throughout the flower, including the vasculature of the sepals, with stronger staining evident immediately below the stigmatic surface (Fig. 5E). Variations in GUS activity were observed in siliques, depending on their developmental stage. Young siliques exhibited very little staining, whereas mature siliques were more intensely stained (data not shown).

Consistent with the effect of wounding on C4H mRNA accumulation (Fig. 3C), C4H-driven GUS activity was induced by wounding. Mature leaves showed relatively little staining in mesophyll and epidermal cells; however, intense staining was observed in these cells after wounding (Fig. 5F). Control leaves that were exposed to X-Gluc immediately after wounding showed no induction in GUS staining (Fig. 5G).

DISCUSSION

Since the cloning of the first plant P450 gene in 1990 (Bozak et al., 1990), the genes encoding a number of plant P450s of diverse function have been identified (Fahrendorf and Dixon, 1993; Holton et al., 1993; Mizutani et al., 1993; Song et al., 1993; Teutsch et al., 1993; Koch et al., 1995; Kraus and Kutchan, 1995; Winkler and Helentjaris, 1995; Bishop et al., 1996; Meyer et al., 1996b; Szekeres et al., 1996). The isolation of these cDNAs has enabled a broad range of new investigations into plant P450s, including a more detailed examination of these catalysts and the characterization of the *cis*-acting elements that regulate their expression.

We have used the high degree of conservation among previously identified C4H sequences to identify the Arabidopsis orthologue from the EST database. Alignment of the

full-length nucleotide sequence and its inferred translation product with sequences available in the GenBank database showed that C4H from Arabidopsis is very similar to C4H from other plants. C4H is encoded at a single locus in Arabidopsis that maps to the lower arm of chromosome 2. Although Phe ammonia-lyase is encoded by a family of three genes in Arabidopsis (Wanner et al., 1995), other enzymes involved in phenylpropanoid metabolism are encoded by single genes. For example, ferulate-5-hydroxylase, chalcone synthase, chalcone isomerase, and dihydroflavonol reductase are all thought to be encoded by single-copy genes, and mutants defective in these genes have been identified as *fah1*, *tt4*, *tt5*, and *tt3*, respectively (Feinbaum and Ausubel, 1988; Chapple et al., 1992; Shirley et al., 1992, 1995). It is noteworthy that no Arabidopsis mutant defective in C4H activity has yet been identified. It is likely that loss-of-function C4H mutants would be lethal due to the impact of such a mutation on vascular development. C4H is a single-copy gene in pea (Frank et al., 1996), whereas it constitutes a small gene family in alfalfa (Fahrendorf and Dixon, 1993), mung bean (Mizutani et al., 1993), and *Catharanthus roseus* (Hotze et al., 1995).

The sequence of the C4H genomic clone revealed that the C4H coding region is interrupted by two introns. In the few plant P450 genomic clones that have been identified to date, the number of introns ranges between two and eight (Mangold et al., 1994; Bishop et al., 1996; Czernic et al., 1996; Szekeres et al., 1996). Like C4H, the Arabidopsis gene encoding ferulate-5-hydroxylase has two introns (K. Meyer and C. Chapple, unpublished results); however, the positions of the introns within the coding regions of these two genes are not similar. The lack of intron/exon similarity between all of these P450 genes is not unexpected, since they share <40% amino acid identity with one another, suggesting that significant evolutionary time has elapsed since these sequences have diverged.

The activity of the phenylpropanoid pathway in Arabidopsis is required in many plant tissues for the synthesis of lignin, seed coat pigments, and soluble metabolites such as sinapate esters and flavonoids (Chapple et al., 1994). To determine whether the tissue specificity of C4H expression is correlated with the accumulation of these products, RNA blots and C4H promoter-driven GUS activity were used to quantify C4H expression in various tissues (Jefferson et al., 1987). Overall, the expression pattern observed in GUS transformants paralleled the expression of C4H as determined by RNA blot hybridization analysis, which indicated that C4H was expressed in all of the plant tissues examined. The role of C4H in the production of the monolignols required for lignification is reflected by the high level of C4H mRNA in stems and the strong GUS staining in the vascular tissue of stems and leaves. The diffuse GUS activity in the cotyledons and primary leaves is consistent with the role of C4H in the accumulation of UV-protective sinapate esters and flavonoids in the epidermis and mesophyll. C4H mRNA is also abundant in siliques, where C4H activity is required for the synthesis of these metabolites. The high level of C4H expression in roots is consistent with previous studies examining the expression of other phenylpropanoid genes such as Phe ammonia-lyase (Wanner et al., 1995), hydroxycinnamoyl CoA ligase (Lee et al., 1995), chalcone synthase (Schmid et al., 1990), and ferulate-5-hydroxylase (J. Cusumano and C. Chapple, unpublished results). Likewise, the intense C4H promoter-driven GUS activity in roots is similar to that observed in studies where the Arabidopsis PAL1 and PAL2 promoters and the parsley hydroxycinnamoyl CoA ligase promoter directed a high level of GUS expression throughout the root and vascular tissues of transgenic Arabidopsis (Ohl et al., 1990; Hatton et al., 1995; Lee et al., 1995). In contrast, chalcone synthase, which catalyzes the first committed step in the flavonoid biosynthetic pathway, was weakly expressed in stems, as compared with roots (Schmid et al., 1990). The reason for the high levels of expression directed by these phenylpropanoid promoters in roots remains open to speculation given that Arabidopsis roots are not known to produce high levels of soluble phenylpropanoid derivatives, nor are they heavily lignified.

Phenylpropanoid pathway gene expression has been shown to be induced by an array of biotic and abiotic factors (Baron and Zambryski, 1995; Dixon and Paiva, 1995), and *cis*-acting elements that are required for response to these stimuli have been identified within a number of their respective promoters (Lois et al., 1989; Lawton et al., 1990; Ohl et al., 1990; Logemann et al., 1995). For example, light plays a pivotal role in the expression of several phenylpropanoid genes (Feinbaum and Ausubel, 1988; Lois et al., 1989; Ohl et al., 1990; Douglas et al., 1991; Kubasek et al., 1992), and our data indicate that C4H is similarly regulated. This finding may account for the previous observations that C4H enzymatic activity increases in etiolated pea seedlings after brief exposure to white light (Russell, 1971) and specifically in response to red and blue light (Beneviste et al., 1978). The light dependence of C4H expression is further demonstrated by the observation that, like chalcone synthase mRNA (Chory et al., 1989; Chory and Peto, 1990), C4H mRNA is accumulated to light-grown levels in dark-grown *det1–1* seedlings. The accumulation of C4H message in dark-grown *det1–1* seedlings would be expected given that they accumulate anthocyanins. The occurrence within the C4H promoter of two G-box-like sequences, which have previously been identified in light-responsive promoters (Giuliano et al., 1988), may account for this property of C4H expression.

C4H activity and/or gene expression has also been shown to be induced by elicitors, pathogens, and wounding (Beneviste et al., 1977; Fahrendorf and Dixon, 1993; Buell and Somerville, 1995; Logemann et al., 1995; Frank et al., 1996). We have found that wounding of Arabidopsis leaves leads to an increase in C4H mRNA accumulation and a localized high level of GUS expression around the wound site in C4H-GUS transgenics. In keeping with these observations, we have identified three box L/AC-I/AC-III/box 1/box 2 homologs (Ohl et al., 1990; da Costa e Silva et al., 1993; Hatton et al., 1995) within the C4H promoter. These elements have previously been shown to be correlated with light-, wound-, and elicitor-inducibility of phenylpropanoid promoters. We have also identified an AC-

rich box-3 homolog 9 bp upstream of the TATA box. This regulatory element has been reported to be correlated with elicitor-inducibility in the bean CHS15 promoter (Lawton et al., 1990) and is also present in the Arabidopsis PAL1 promoter (Ohl et al., 1990). In view of our results indicating that the Arabidopsis C4H promoter is activated in response to wounding, and that C4H message accumulation is induced by both light and pathogen attack (Buell and Somerville, 1995), it will be important to test the hypothesis that these regulatory sequences are involved in C4H regulation in Arabidopsis.

Exogenous application of phenylpropanoid pathway intermediates has been shown to affect the levels of Phe ammonia-lyase, C4H, and chalcone synthase and the expression of their corresponding genes (Lamb and Rubery, 1976; Bolwell et al., 1988; Orr et al., 1993). The presence of H-box and G-box elements was shown to be necessary for this response in the bean CHS15 promoter (Loake et al., 1992). The identification of similar motifs within the C4H promoter and the availability of Arabidopsis phenylpropanoid mutants (Chapple et al., 1992; Shirley et al., 1995) prompted us to investigate whether C4H expression was affected in any of these mutant lines. In most of the mutants only small differences in C4H expression were observed, suggesting that either C4H is not regulated in response to pathway intermediate pool sizes, or that these mutations do not lead to changes in these pools that are sufficiently large to have an impact. In contrast, C4H mRNA was almost doubled in seedlings of the *tt8* mutant. The TT8 locus has been suggested to encode a regulatory protein that affects dihydroflavonol reductase expression (Shirley et al., 1995). It is interesting that the *tt8* mutant has increased levels of C4H mRNA in view of the fact that this mutation has an opposite effect on dihydroflavonol reductase mRNA accumulation (Shirley et al., 1995). In addition, C4H expression is unaffected in the *tt3* mutant, which is defective in the gene encoding dihydroflavonol reductase. These data would suggest that the *TT8* gene product may act directly on C4H expression, rather than via modulation of dihydroflavonol reductase expression. The mechanism by which C4H expression is altered in this mutant will require both the further dissection of the C4H promoter and possibly the cloning of the *TT8* gene.

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LITERATURE CITED

Baron C, Zambryski PC (1995) The plant response in pathogenesis, symbiosis, and wounding: variations on a common theme? Annu Rev Genet **29:** 107–129

Beneviste I, Salaun JP, Durst F (1977) Wounding-induced cinnamic acid hydroxylase in Jerusalem artichoke tuber. Phytochemistry **16:** 69–73

Beneviste I, Salaun JP, Durst F (1978) Phytochrome-mediated regulation of a monooxygenase hydroxylating cinnamic acid in etiolated pea seedlings. Phytochemistry **17:** 359–363

Bent AF, Kunkel BN, Dahlbeck D, Brown KL, Schmidt R, Giraudat J, Leung J, Staskawicz BJ (1994) *RPS2* of *Arabidopsis thaliana:* a leucine-rich repeat class of plant disease resistance genes. Science **265:** 1856–1860

Bishop GJ, Harrison K, Jones JDG (1996) The tomato *dwarf* gene isolated by heterologous transposon tagging encodes the first member of a new cytochrome P450 family. Plant Cell **8:** 959–969

Bolwell GP, Bozak K, Zimmerlin A (1994) Plant cytochrome P450. Phytochemistry **37:** 1492–1506

Bolwell GP, Dixon RA (1986) Membrane-bound hydroxylases in elicitor-treated bean cells. Rapid induction of the synthesis of prolyl hydroxylase and a putative cytochrome P-450. Eur J Biochem **159:** 163–169

Bolwell GP, Mavandad M, Millar DJ, Edwards KJ, Schuch W, Dixon RA (1988) Inhibition of mRNA levels and activities by trans-cinnamic acid in elicitor-induced bean cells. Phytochemistry **27:** 2109–2117

Bozak KR, Yu H, Sirevåg R, Christoffersen RE (1990) Sequence analysis of ripening-related cytochrome P-450 cDNAs from avocado fruit. Proc Natl Acad Sci USA **87:** 3904–3908

Brown JWS, Smith P, Simpson CG (1996) Arabidopsis consensus intron sequences. Plant Mol Biol (in press)

Buell CR, Somerville SC (1995) Expression of defense-related and putative signaling genes during tolerant and susceptible interactions of *Arabidopsis* with *Xanthomonas campestris* pv. *campestris*. Mol Plant-Microbe Interact **8:** 435–443

Caldwell MM, Robberecht R, Flint SD (1983) Internal filters: prospects for UV-acclimation in higher plants. Physiol Plant **58:** 445–450

Chapple CCS, Shirley BW, Zook M, Hammerschmidt R, Somerville SC (1994) Secondary metabolism in Arabidopsis. *In* C Somerville, E Meyerowitz, eds, Arabidopsis. Cold Spring Harbor Laboratory Press, Plainview, NY, pp 989–1030

Chapple CCS, Vogt T, Ellis BE, Somerville CR (1992) An Arabidopsis mutant defective in the general phenylpropanoid pathway. Plant Cell **4:** 1413–1424

Chory J, Peto C (1990) Mutations in the DET1 gene affect cell-type-specific expression of light-regulated genes and chloroplast development in *Arabidopsis*. Proc Natl Acad Sci USA **87:** 8776–8780

Chory J, Peto C, Feinbaum R, Pratt L, Ausubel F (1989) *Arabidopsis thaliana* mutant that develops as a light-grown plant in the absence of light. Cell **58:** 991–999

Cooke R, Raynal M, Laudié M, Grellet F, Delseny M, Morris P-C, Guerrier D, Giraudat J, Quigley F, Clabault G, and others (1996) Further progress towards a catalogue of all *Arabidopsis* genes: analysis of a set of 5000 non-redundant ESTs. Plant J **9:** 101–124

Czernic P, Moliere F, Marco Y (1996) Structural organization of a genomic clone encoding a cytochrome P450-dependent monooxygenase from *Nicotiana tabacum* (accession no. X96784) (PGR 96–034). Plant Physiol **111:** 652

da Costa e Silva O, Klein L, Schmelzer E, Trezzini GF, Hahlbrock K (1993) BPF-1, a pathogen-induced DNA-binding protein involved in the plant defense response. Plant J **4:** 125–135

Dixon R, Paiva N (1995) Stress-induced phenylpropanoid metabolism. Plant Cell **7:** 1085–1097

Douglas CJ, Hauffe KD, Ites-Morales ME, Ellard M, Paszkowski U, Hahlbrock K, Dangl JL (1991) Exonic sequences are required for elicitor and light activation of a plant defense gene, but promoter sequences are sufficient for tissue specific expression. EMBO J **10:** 1767–1775

Durst F, O'Keefe DP (1995) Plant cytochromes P450: an overview. Drug Metab Drug Interact **12:** 171–187

Fahrendorf T, Dixon RA (1993) Stress responses in alfalfa (*Medicago sativa* L.) XVIII: molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450. Arch Biochem Biophys **305:** 509–515

Feinbaum RL, Ausubel FM (1988) Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol Cell Biol **8:** 1985–1992

Feinbaum RL, Storz G, Ausubel FM (1991) High intensity and blue light regulated expression of chimeric chalcone synthase genes in transgenic *Arabidopsis thaliana* plants. Mol Gen Genet **226:** 449–456

Feuillet C, Lauvergeat V, Deswarte C, Pilate G, Boudet A, Grima-Pettenati J (1995) Tissue- and cell-specific expression of a cinnamyl alcohol dehydrogenase in transgenic poplar plants. Plant Mol Biol **27:** 651–667

Frank MR, Deyneka JM, Schuler MA (1996) Cloning of wound-induced cytochrome P450 monooxygenases expressed in pea. Plant Physiol **110:** 1035–1046

Gabriac B, Werck-Reichhart D, Teutsch H, Durst F (1991) Purification and immunocharacterization of a plant cytochrome P450: the cinnamic acid 4-hydroxylase. Arch Biochem Biophys **288:** 302–309

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res **50:** 151–158

Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnik PA, Cashmore AR (1988) An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc Natl Acad Sci USA **85:** 7089–7093

Goldsbrough PB, Cullis CA (1981) Characterization of the genes for ribosomal RNA in flax. Nucleic Acids Res **9:** 1301–1309

Hatton D, Sablowski R, Yung M, Smith C, Schuch W, Bevan M (1995) Two classes of *cis* sequences contribute to tissue-specific expression of a PAL2 promoter in transgenic tobacco. Plant J **7:** 859–876

Hauffe KD, Paszkowski U, Schulze-Lefert P, Hahlbrock K, Dangl JL, Douglas CJ (1991) A parsley 4CL-1 promoter fragment specifies complex expression patterns in transgenic tobacco. Plant Cell **3:** 435–443

Holton TA, Brugliera F, Lester DR, Tanaka Y, Hyland CD, Menting JGT, Lu C-Y, Farcy E, Stevenson TW, Cornish EC (1993) Cloning and expression of cytochrome P450 genes controlling flower colour. Nature **366:** 276–279

Hotze M, Schroder G, Schroder J (1995) Cinnamate 4-hydroxylase from *Catharanthus roseus,* and a strategy for the functional expression of plant cytochrome P450 proteins as translational fusions with P450 reductase in *Escherichia coli.* FEBS Lett **374:** 345–350

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **6:** 3901–3907

Koch BM, Sibbesen O, Halkier BA, Svendsen I, Møller BL (1995) The primary sequence of cytochrome $P450_{TYR}$, the multifunctional *N*-hydroxylase catalyzing the conversion of L-tyrosine to *p*-hydroxyphenylacetaldehyde oxime in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor* (L.) Moench. Arch Biochem Biophys **323:** 177–186

Kraus PFX, Kutchan TM (1995) Molecular cloning and heterologous expression of a cDNA encoding berbamunine synthase, a C-O phenol-coupling cytochrome P450 from the higher plant *Berberis stolonifera.* Proc Natl Acad Sci USA **92:** 2071–2075

Kubasek WL, Shirley BW, McKillop A, Goodman HM, Briggs W, Ausubel FM (1992) Regulation of flavonoid biosynthetic genes in germinating Arabidopsis seedlings. Plant Cell **4:** 1229–1236

Lamb CJ, Rubery PH (1976) Phenylalanine ammonia-lyase and cinnamic acid 4-hydroxylase: product repression of the level of enzyme activity in potato tuber discs. Planta **130:** 283–290

Landry LG, Chapple CCS, Last R (1995) Arabidopsis mutants lacking phenolic sunscreens exhibit enhanced ultraviolet-B injury and oxidative damage. Plant Physiol **109:** 1159–1166

Lawton MA, Clouse SD, Lamb CJ (1990) Glutathione-elicited changes in chromatin structure within the promoter of the defense gene chalcone synthase. Plant Cell Rep **8:** 561–564

Lee D, Ellard M, Wanner LA, Davis KR, Douglas CJ (1995) The *Arabidopsis thaliana* 4-coumarate:CoA ligase (4CL) gene: stress and developmentally regulated expression and nucleotide sequence of its cDNA. Plant Mol Biol **28:** 871–884

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Ann Rev Plant Physiol Plant Mol Biol **41:** 455–496

Li J, Ou-Lee T-M, Raba R, Amundson R, Last RL (1993) Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell **5:** 171–179

Lister C, Dean C (1993) Recombinant inbred lines for mapping RFLP and phenotypic markers in *Arabidopsis thaliana.* Plant J **4:** 745–750

Loake GJ, Choudhary AD, Harrison MJ, Mavandad M, Lamb CJ, Dixon RA (1991) Phenylpropanoid pathway intermediates regulate transient expression of a chalcone synthase gene promoter. Plant Cell **3:** 829–840

Loake GJ, Faktor O, Lamb CJ, Dixon RA (1992) Combination of H-box [CCTACC$(N)_7$CT] and G-box (CACGTG) cis elements is necessary for feed-forward stimulation of a chalcone synthase promoter by the phenylpropanoid-pathway intermediate *p*-coumaric acid. Proc Natl Acad Sci USA **89:** 9230–9234

Logemann E, Parniske M, Hahlbrock K (1995) Modes of expression and common structural features of the complete phenylalanine ammonia-lyase family in parsley. Proc Natl Acad Sci USA **92:** 5905–5909

Lois R, Dietrich A, Hahlbrock K, Schulz W (1989) A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. EMBO J **8:** 1641–1648

Mangold U, Eichel J, Batschauer A, Lanz T, Kaiser T, Spangenberg G, Werck-Reichhart D, Schröder J (1994) Gene and cDNA for plant cytochrome P450 proteins (CYP72 family) from *Catharanthus roseus,* and transgenic expression of the gene and a cDNA in tobacco and *Arabidopsis thaliana.* Plant Sci **96:** 129–136

Meyer K, Benning G, Grill E (1996a) Cloning of plant genes based on genetic map location. *In* AH Paterson, ed, Genome Mapping in Plants. Academic Press, New York; Landes Bioscience Publishers, Austin, TX, pp 137–154

Meyer K, Cusumano JC, Somerville CR, Chapple CCS (1996b) Ferulate-5-hydroxylase from *Arabidopsis thaliana* defines a new family of cytochrome P450-dependent monooxygenases. Proc Natl Acad Sci USA **93:** 6869–6874

Mizutani M, Ward E, DiMaio J, Ohta D, Ryals J, Sato R (1993) Molecular cloning and sequencing of a cDNA encoding mung bean cytochrome P450 ($P450_{C4H}$) possessing cinnamate 4-hydroxylase activity. Biochem Biophys Res Commun **190:** 875–880

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant **15:** 473–497

Newman T, de Bruijn FJ, Green P, Keegstra K, Kende H, McIntosh L, Ohlrogge J, Raikhel N, Somerville S, Thomashow M, Retzel E, Somerville C (1994) Genes galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. Plant Physiol **106:** 1241–1255

Nicholson RL, Hammerschmidt R (1992) Phenolic compounds and their role in disease resistance. Annu Rev Phytopathol **30:** 369–389

Ohl S, Hedrick SA, Chory J, Lamb CJ (1990) Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis.* Plant Cell **2:** 837–848

O'Malley DM, Whetten R, Bao W, Chen C-L, Sederoff RR (1993) The role of laccase in lignification. Plant J **4:** 751–757

Orr J, Edwards R, Dixon RA (1993) Stress responses in alfalfa (*Medicago sativa* L.) XIV. Changes in the levels of phenylpropanoid pathway intermediates in relation to regulation of L-phenylalanine ammonia-lyase in elicitor-treated cell-suspension cultures. Plant Physiol **101:** 847–856

Rogers SO, Bendich AJ (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. Plant Mol Biol **5:** 69–76

Russell D (1971) The metabolism of aromatic compounds in higher plants. X. Properties of the cinnamic acid 4-hydroxylase of pea seedlings and some aspects of its metabolic and developmental control. J Biol Chem **246:** 3870–3878

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning. A laboratory manual, Ed 2. Cold Spring Harbor Laboratory Press, Plainview, NY

Schmid J, Doerner PW, Clouse SD, Dixon RA, Lamb CJ (1990) Developmental and environmental regulation of a bean chalcone synthase promoter in transgenic tobacco. Plant Cell **2:** 619–631

Schuler, MA (1996) Plant cytochrome P450 monooxygenases. Crit Rev Plant Sci **15:** 235–284

Shirley BW, Hanley S, Goodman HM (1992) Effects of ionizing radiation on a plant genome: analysis of two Arabidopsis transparent testa mutations. Plant Cell **4:** 333–347

Shirley BW, Kubasek WL, Storz G, Bruggemann E, Koornneef M, Ausubel FM, Goodman HM (1995) Analysis of Arabidopsis mutants deficient in flavonoid biosynthesis. Plant J **8:** 659–671

Song W-C, Funk CD, Brash AR (1993) Molecular cloning of an allene oxide synthase: a cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. Proc Natl Acad Sci USA **90:** 8519–8523

Stomp A (1992) Histochemical localization of β-glucuronidase. *In* SR Gallagher, ed, GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression. Academic Press, San Diego, CA 103–113

Szczesna-Skorupa E, Straub P, Kemper B (1993) Deletion of a conserved tetrapeptide, PPGP, in P450 2C2 results in loss of enzymatic activity without a change in its cellular location. Arch Biochem Biophys **304:** 170–175

Szekeres M, Németh K, Koncz-Kálmán Z, Mathur J, Kauschmann A, Altmann T, Rédei GP, Nagy F, Schell J, Koncz C (1996) Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. Cell **85:** 171–182

Teutsch HG, Hasenfratz MP, Lesot A, Stoltz C, Garnier J-M, Jeltsch J-M, Durst F, Werck-Reichhart D (1993) Isolation and sequence of a cDNA encoding the Jerusalem artichoke cinnamate 4-hydroxylase, a major plant cytochrome P450 involved in the general phenylpropanoid pathway. Proc Natl Acad Sci USA **90:** 4102–4106

Von Wachenfeldt C, Johnson EF (1995) Structures of eukaryotic cytochrome P450 enzymes. *In* PR Ortiz de Montellano, ed, Cytochrome P450: Structure, Mechanism, and Biochemistry, Ed 2. Plenum Press, New York, pp 183–223

Wanner L, Li G, Ware D, Somssich, Davis K (1995) The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant Mol Biol **27:** 327–338

Winkler RG, Helentjaris T (1995) The maize *dwarf3* gene encodes a cytochrome P450-mediated early step in gibberellin biosynthesis. Plant Cell **7:** 1307–1317

Yamazaki S, Sato K, Suhara K, Sakaguchi M, Mihara K, Omura T (1993) Importance of the proline-rich region following signal-anchor sequence in the formation of correct conformation of microsomal cytochrome P-450s. J Biochem **114:** 652–657

Zambryski P, Joos H, Genetello C, Leemans J, van Montagu M, Schell J (1983) Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J **2:** 2143–2150